AUTOREGULATION OF THE GLIAL GENE *REVERSED POLARITY* IN *DROSOPHILA MELANOGASTER*

by
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ABSTRACT

Autoregulation is the process where an encoded protein is able to bind to and positively or negatively regulate its own expression. Autoregulatory loops are crucial for sustained gene expression, and such loops have been demonstrated to be important for development in organisms ranging from *Danio rerio* to *Arabidopsis thaliana* and *Drosophila melanogaster.* The cells of the nervous system arise from progenitor cells that eventually adopt one of two fates: neuronal or glial. This decision is controlled by *glial cells missing*; however *glial cells missing* is expressed briefly at the beginning development. The glial gene *repo* is a gene that is activated by *gcm.* Following activation, *repo* is expressed for the rest of the life span of the organism. In this study, we present evidence that Repo is capable of sustaining its own expression through a positive autoregulatory mechanism.

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LIST OF ABBREVIATIONS

CRD	*cis*-regulatory DNA
Repo	Reversed polarity protein
repo	*reversed polarity* gene
Gcm	Glial cells missing protein
gcm	*glial cells missing* gene
Ubx	Ultrabithorax protein
ubx	*ultrabithorax* gene
dpp	*decapentaplegic* gene
LAR II	Luciferase Assay Reagent II
RBS	Repo Binding Site

CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1 Overview of Eukaryotic Gene Regulation

The expression of genes and their protein products can be regulated at several steps. These include regulation of transcription, mRNA, translation, and post-translational protein modifications. The enhancer and promoter regions of a gene control transcription, with transcription factors binding the enhancer region in order to bring RNA Polymerase II to the promoter. RNA Polymerase II then transcribes the gene into a pre-mRNA transcript, which is then modified by splicing out of introns, along with the addition of a poly-A tail and a 7-methylguanosine 5' cap. Following these modifications, the mature mRNA is exported from the nucleus to the cytoplasm where it is translated into protein.

Some genes are held in a ready state, with RNA Polymerase II temporarily paused at the promoter. This pause, termed promoter-proximal pausing, allows crucial genes to be transcribed quickly (1). Heat shock proteins are classic example of genes that have a high percentage of the promoter-proximal downstream region occupied by a paused RNA Polymerase II (2).

Transcriptional regulation is maintained by the affinity of RNA Polymerase II for the promoter. The first mechanism for modulation of this Promoter/RNA Polymerase II affinity is the homology of any given promoter to the consensus sequence. The second mechanism used to bring RNA Polymerase II to the promoter is the presence of

cis-regulatory DNA binding by transcription factors. These *cis*-regulatory domains (CRD), which contain enhancer elements, can be found thousands of base pairs upstream of the gene start site. It is believed that this CRD is able to interact with the promoter through a process termed looping out. Through this mechanism, transcription factors bound to CRD thousands of base pairs upstream are able to interact with the promoter.

1.2 Defininig Mechanisms of Transcriptional Autoregulation

Transcriptional autoregulation is a phenomenon that is present in organisms spanning from *Drosophila melanogaster* to *Arabidopsis thaliania* to humans. In their 2009 article Crews and Pearson divide autoregulation into several different categories, including direct positive autoregulation, feed forward positive autoregulation, indirect positive autoregulation, and direct negative autoregulation.

In direct positive autoregulation, an initial transcriptional activator initiates the transcription and translation of a gene (Gene A) encoding a protein capable of binding its own enhancer region positively regulating transcription (3). The homeobox gene *reversed polarity* (*repo*) is one example of a gene that is controlled by a direct positive autoregulatory pathway. Here, Glial cells missing (Gcm) is capable of binding the CRD of the *repo* gene (4). More specifically, the N-terminal region of Gcm was shown to bind eleven (A/G)CCCGCAT sequences found in the upstream region of the *repo* gene (4). However, *gcm* is only expressed transiently during development, while *repo* is expressed throughout the life of *Drosophila melanogaster*. Therefore, a mechanism must exist to sustain *repo* expression once *gcm* is no longer expressed at a sufficient level to drive *repo* transcription.

Feed forward positive autoregulation is similar to direct positive autoregulation, differing in the number of downstream genes effected. In this mechanism, Gene A encodes a protein that is capable of binding not only its own enhancer region, but the enhancer region of other genes as well. This mechanism is able to initiate the transcription of multiple genes utilizing only one initial transcriptional activator.

The maintenance of gene expression can also be controlled through an indirect positive autoregulatory mechanism that is mediated through cell signaling. Crews and Pearson provide the example of Ultrabithorax (Ubx) maintenance in the parasegments of *Drosophila melanogaster.* In their paper, they show *Ubx* being transcriptionally activated by some initial activating transcription factor. Ubx protein is then able to activate *decapentaplegic (dpp)* expression in parasegment 7. Dpp protein is then able to regulate *wingless* (*wg*) expression in parasegment 8. Wg protein is then able to feedback and signal parasegment 7, increasing *ubx* expression via the Tcf transcription factor.

Finally, direct negative autoregulation is a mechanism to either maintain gene expression at a consistent level or to abolish gene expression entirely. An example of direct negative autoregulation can be found in the development of the eye lens in chick. Pre-lens ectoderm explants were cultured in the presence of either Noggin or BMP4/7. Noggin, a known BMP antagonist was shown to significantly increase the levels of *Bmp2,* with a lesser increase seen in *Bmp4. Bmp7* expression was not altered. Explants cultured in media containing a BMP4/7 mixture resulted in a significant decrease in *Bmp2* and *Bmp4,* but increased *Bmp7* (5). Negative autoregulation of BMP expression serves as a mechanism to maintain stable expression of BMPs, as suggested by the

observation that exogenous BMPs decrease *Bmp4* and *7* transcripts, while BMP antagonist (Noggin) addition results in increased *Bmp4* and *7* transcripts.

1.3 Gliogenesis in *Drosophila melanogaster*

As mentioned previously, glial cells, along with neurons, differentiate from progenitor cells. The gene *glial cells missing* (*gcm*) is a master regulator of cell fate in the nervous system, functioning to push progenitor cells to a glial fate if expressed. If *gcm* is not expressed in a progenitor cell, that cell adopts a neuronal lineage. In *gcm* loss-of-function embryos, presumptive glial cells differentiate into neurons. *Drosophila* embryos ectopically expressing *gcm* exhibit the opposite phenotype, with nearly all neurons being transformed to glia (6). These experiments show *gcm* to be a binary genetic switch controlling glial vs. neuronal determination.

Although *gcm* is considered a master regulator of glial cell development, it is expressed only transiently in the developing embryo. It has been shown that Polycomb controls this transient expression through a *gcm* repressive mechanism (7). As *gcm* persists for a short time during development, downstream genes must be activated to maintain glial fate. As previously mentioned, the *repo* gene has been shown to be a downstream target of Gcm, with Gcm binding the CRD of *repo* to activate *repo* transcription. Mutations in the *repo* gene do not affect early glial cell formation, but do lead to a reduction in the number of glial cells and increased neuronal cell death. These observations indicate that *repo* is crucial for terminal glial differentiation (8).

If *repo* is crucial to sustain a glial phenotype, then a positive autoregulatory mechanism to maintain *repo* expression is logical. Such a mechanism would include *gcm*

as an initial transcriptional activator of *repo*, with Repo having the ability to bind its own CRD with subsequent activation of transcription. There are three Repo Binding Sites (RBS) in the 4.3 kb upstream of the *repo* gene.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Drosophila melanogaster* S2 Cell Culture

S2 cells were initiated from frozen stocks stored in liquid nitrogen. Cells were cultured in complete Schneider's Media (10% Fetal Bovine Serum (FBS), 5% Penicillin Streptomycin) at 25 °C, were subcultured every three days. Cell counts were performed using Trypan Blue exclusion. Briefly, 10 µL of cell suspension was added to 490 µL of 1X Phosphate Buffered Saline (PBS) and 500 µL of 0.4% Trypan Blue. Trypan Blue stains non-viable cells, allowing a more accurate count of viable cells to be obtained by excluding stained cells from the calculation. The number of cells in the four corner quadrants were tabulated and divided by 4 to obtain the average number of cells per quadrant. The average number of cells was then multiplied by two to account for the Trypan Blue, and again multiplied by 50 to account for the dilution of the cell suspension in PBS. This number was then multiplied by 10,000 to obtain the number of cells per mL.

Cells were subcultured by blowing a stream of media at the monolayer several times to dislodge adherent cells. The cell suspension was transferred to a 15 mL conical tube and spun at 1500 rpm for 5 minutes. Following centrifugation, the pellet was resuspended in 8 mL of fresh Schneider's Media and 2 mL of conditioned media. A sufficient amount of this cell suspension was transferred to a new T-75 flask in order to propagate the cell

2.2 Dual Luciferase Reporter Assays

S2 cells were washed from culture dishes, spun down, resuspended in fresh media. Cells were counted using a hemocytometer and dispensed into a 24 well plate at a concentration of 350,000 cells per well. Cells were allowed to incubate overnight, and were transfected the following morning. Lipofectamine (Invitrogen) mediated transfections were conducted in serum free media for four hours.

Unstimulated wells were transfected with *pacHA*, *pacRenilla*, *UAS-repo*, and the luciferase construct being tested. Stimulated wells were transfected with *pacGal4*, *pacRenilla*, *UAS-repo*, and the luciferase construct being tested. *pacHA* serves as an empty vector that does not express Gal4. Gal4 is a transcriptional activator from yeast that has no known regulatory targets in *Drosophila.* Gal4 acts on the upstream-activating-sequence (UAS) found in *UAS-repo* to initiate transcription of the *repo* gene. The transfection design is laid out if Figure 1. Following the four hour transfection incubation period, the transfection solution was removed and replaced with complete S2 media. Cells were allowed to incubate a further 48 hours, at which point Firefly and Beetle Luciferase were quantified. Cells were lysed in the 24 well plate they were cultured in using 1X Passive Lysis Buffer (PLB). Cell lysates were collected from non-stiumlated and stimulated wells into individual Eppendorf tubes. For each well, one Eppendorf tube containing 50 μL of Luciferase Assay Reagent II (LAR II) was prepared

To quantify the luciferase expression, 20 μL of cell lysate was added to one of the LAR II containing tubes (Figure 2). The tube was placed into a luminometer and the Firefly luminescence was quantified. The same tube was removed from the luminometer and supplemented with 50 μL of Stop-Glo Reagent. Stop Glo quenches the activity of

Firefly luciferase and activates the Renilla luciferase. Following Stop-Glo Reagent, the *Renilla* luciferase activity was measured. Finally, Firefly luciferase activity was normalized to Renilla luciferase activity.

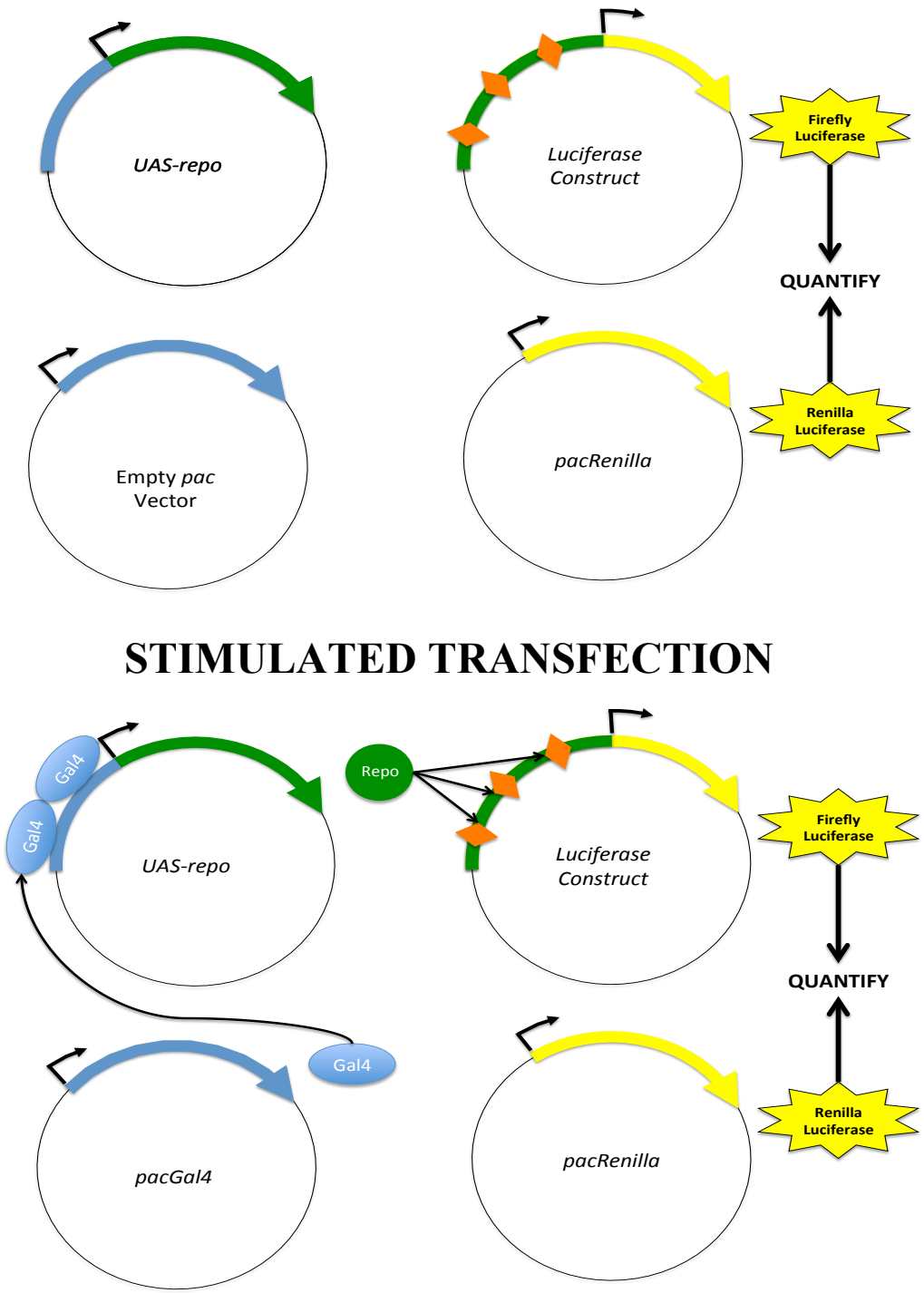

Figure 1: Transfection Design for Non-stimulated and Stimulated Cells.
Non-stimulated cells (A) were transfected with an empty vector along with U*AS-Repo*, a luciferase construct, and *pacRenilla.* Stimulated cells were transfected with a Gal4 expressing plasmid under the control of the actin promoter. Gal4 bind the upstream-activating sequence, triggering *repo* expression. Repo protein then acts on the *repo* CRD in the various luciferase constructs, initiating Firefly Luciferase expression. Firefly Luciferase is normalized to Beetle luciferase expression.

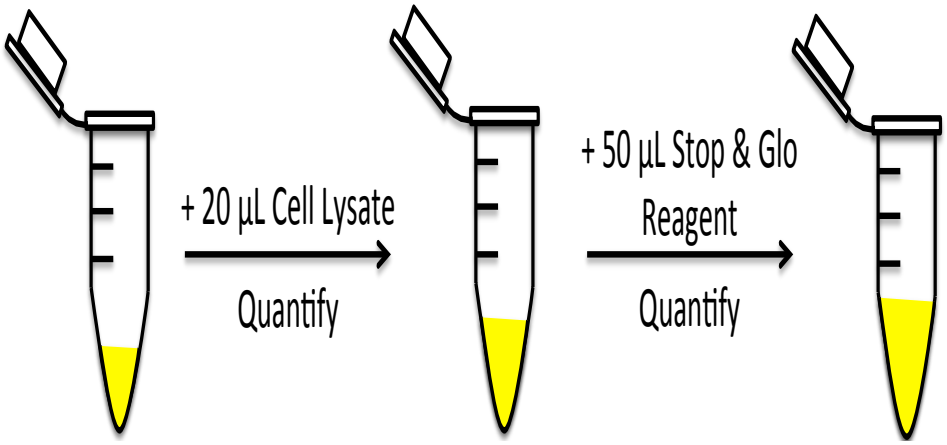

Figure 2: Quantification of Firefly and *Renilla* Luciferases.
The catalytic activities of Firefly and Renilla luciferases were quantified using a luminometer. Quantification of these two luciferases allowed inferences to be made regarding the ability of Repo to autoregulate its own expression.

CHAPTER 3

RESULTS

The results of the luciferase assays indicate that ectopic expression of Repo protein in cultures S2 cells results in an increase in Firefly luciferase expression. The magnitude of the increase was dependent upon the *repo-luciferase* construct that *UAS-repo* was co-transfected with. *pacGal4* supplied Gal4 protein, necessary to activate *repo* expression in the *UAS-repo* construct. Expressed Repo was then free to bind differing fragments of the CRD of *repo*. These CRD fragments were fused to a *luciferase* reporter.

The luciferase assays show that Repo protein expression is capable of increasing the level of luciferase activity. Furthermore, this increase appears to be dependent upon which *luciferase* construct was used in the transfection. The wild type CRD resulted in a ~7 fold increase in luciferase activity over the control. Interestingly, removal of RBS1 and RBS2 resulted in a ~9.5 fold increase over the control, while a construct lacking RBS1 but maintaining RBS2 and RBS3 generated only a 4.6 fold increase.

Mutation of RBS1 was shown to decrease luciferase activity. When compared to repo -4.3/-2.3, repo -4.3/-2.3 RBS1 saw a loss of ~4 fold changes over the control, with repo -4.3/-2.3 RBS showing ~5.2 fold change in luciferase activity. This result indicates that RBS is the most important of the three RBS. It should be noted that statistical significance between the constructs has yet to be determined.

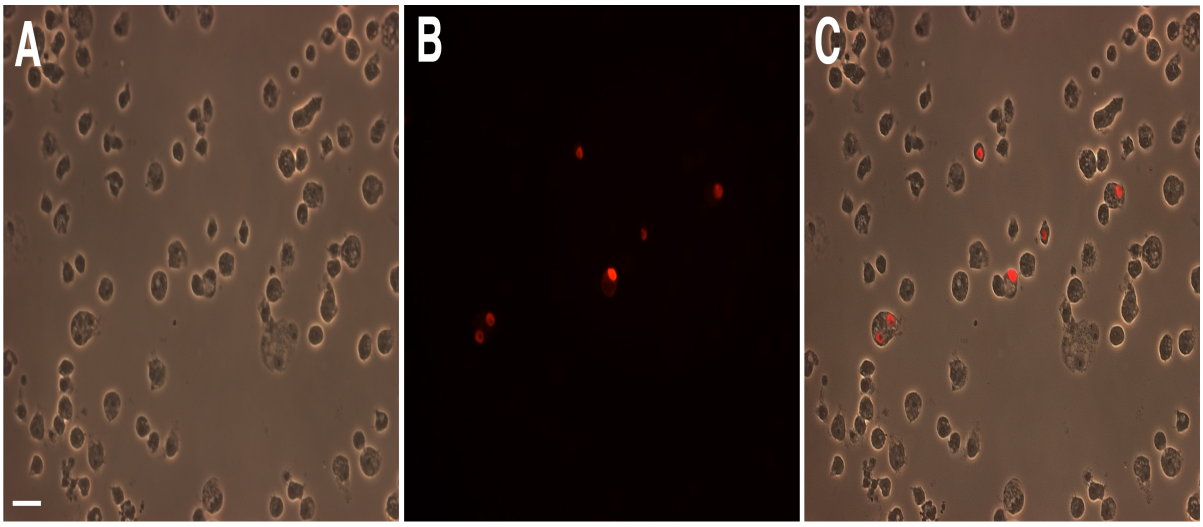

Figure 3: Antibody Staining of Repo in Cultured S2 Cells.
A) Fixed S2 cells visualized utilizing phase contrast microscopy. B) The same field of cells visualized using fluorescence microscopy to detect Repo antibody staining. Repo is only expressed in cells that have undergone a successful transfection. C) A merged image shows Repo expression localization. Scale bar: 20 μm.

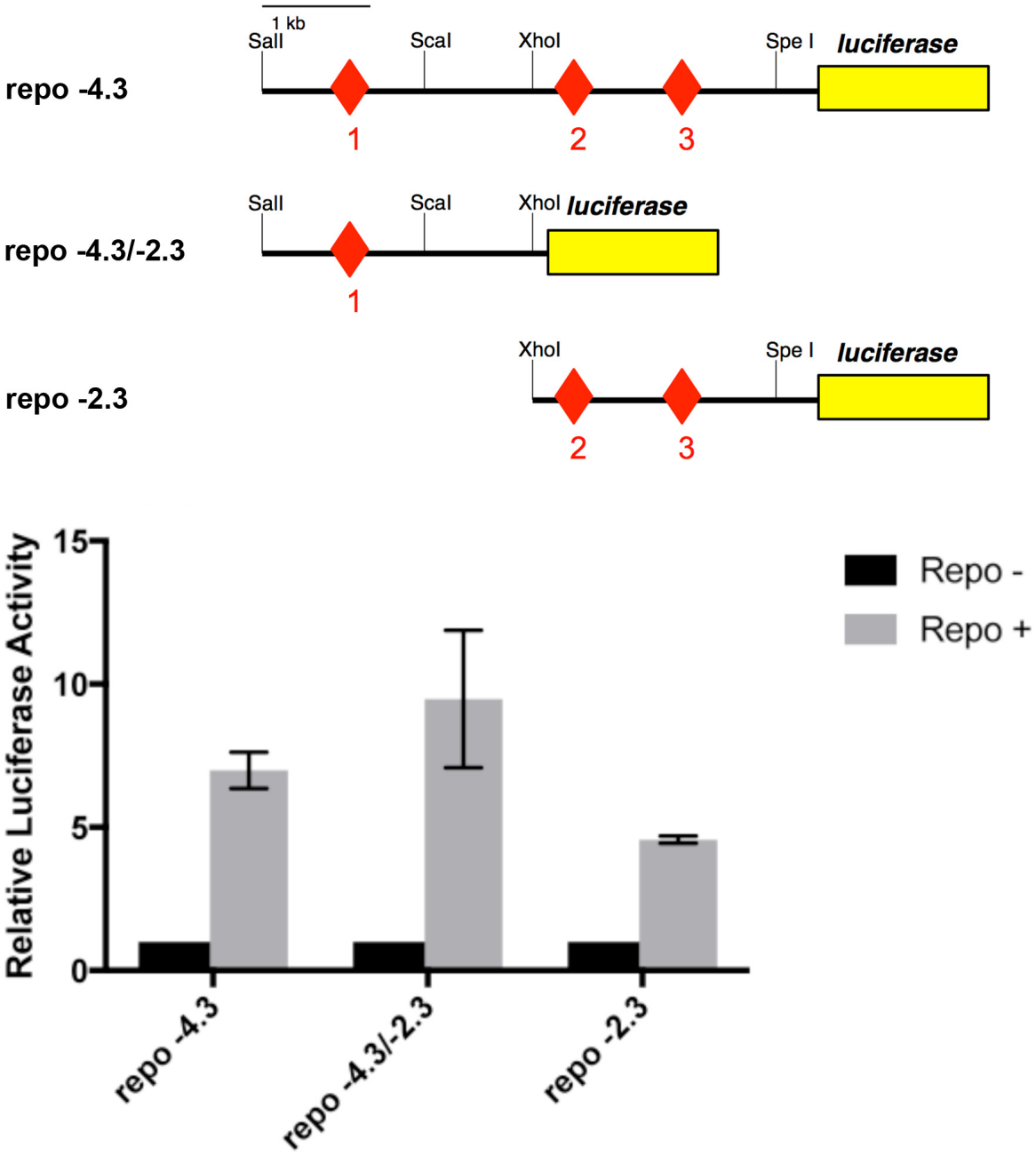

Figure 4. Luciferase activity varies between constructs.
Different fragments of CRD were fused to a luciferase reporter gene. Of these reporters, repo -4.3/-2.3 showed the greatest fold change in luciferase activity over the control. Repo -2.3 showed the lowest fold change in luciferase activity, with repo -4.3 intermediate.

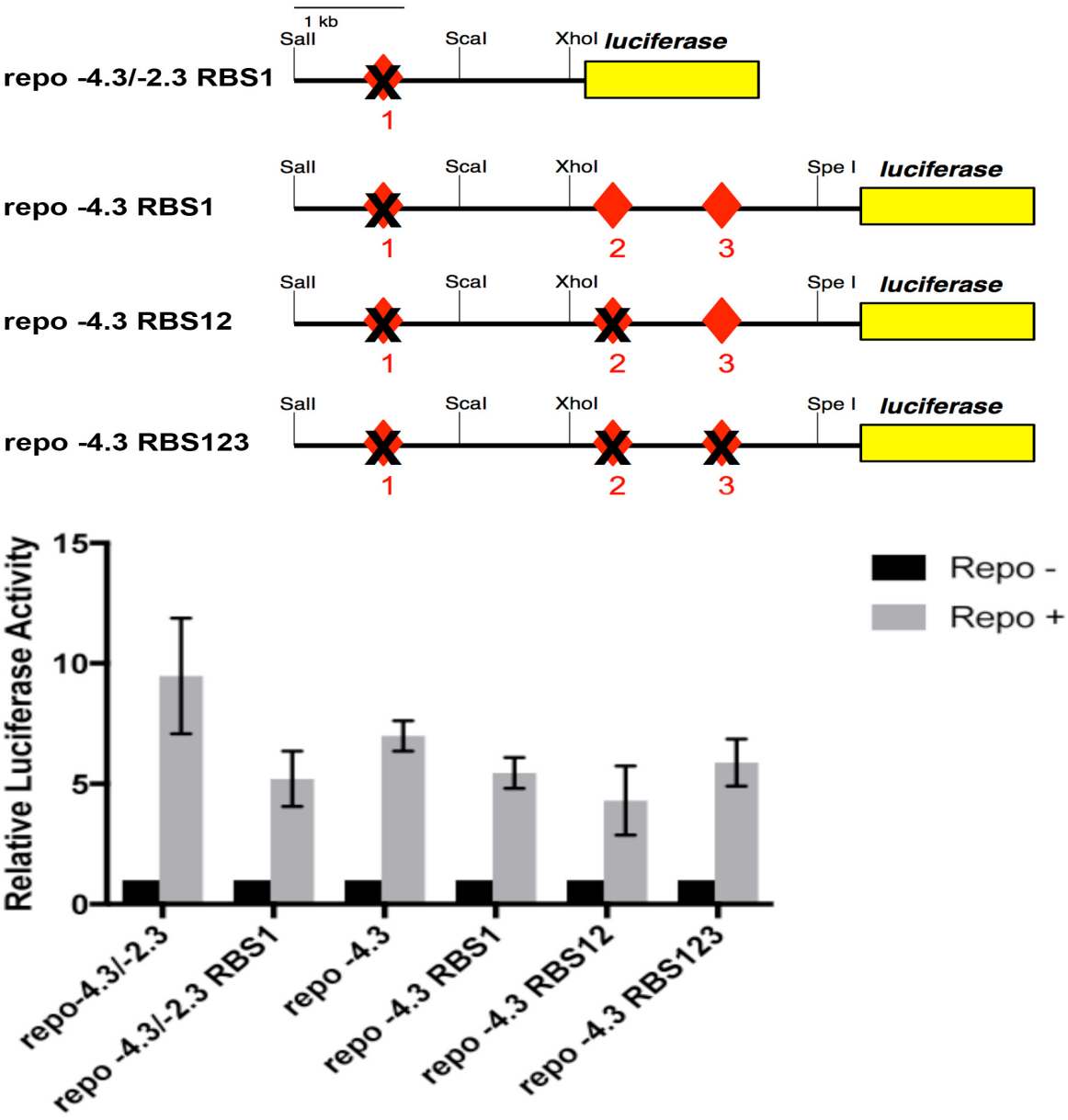

Figure 5. Mutation of RBS1 leads to a decrease in luciferase activity.
Fragments of the *repo* CRD with mutation to the RBS were fused to a luciferase reporter. Mutations of RBS1 lead to a decrease in luciferase activity when compared to repo -4.3/-23. This indicates that RBS1 is the most important site of Repo interaction.

CHAPTER 4

DISCUSSION

The results presented here suggest that Repo is capable of maintaining its own expression via a positive autoregulatory interaction with its own CRD. Furthermore, mutation of RBS1 decreases *repo* expression to a greater degree than mutation of RBS2 or RBS3. This indicates that RBS1 may be the most important of the three canonical Repo binding sites.

It is also possible that RBS 2 and 3 may serve to prevent an excess of Repo expression through a repressive mechanism. When only RBS1 is present, there is a higher fold change in luciferase activity than when all three RBSs are present. The repressive qualities of RBS 2 and 3 could be investigated by creating a luciferase construct containing RBS1 with RBS 2 and 3 mutated. The data from this construct could be compared to repo -4.3. If the construct with canonical RBS1 and mutated RBS2/3 showed an increased luciferase activity over the wild type, then it would be worth further investigation of the repressive effects of RBS 2 and 3.

From this study, autoregulation seems to be a feasible mechanism for the sustained expression of *repo*. However, it still remains to be determined if the conserved 5'CAATTA3' sequence is actually bound by Repo. This could be demonstrated through a gel shift assay in which oligos containing the RBS, along with purified Repo, are run through a gel. If there is a Repo/DNA interaction, then the protein and DNA complex should migrate through the gel slower.

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